Artificial
Organs



# Three-Dimensional Geometry of Honeycomb Collagen Promotes Higher Beating Rate of Myocardial Cells in Culture

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**Abstract:** Myocardial cells were isolated from newborn rats, cultured on a novel three-dimensional (3-D) honeycomb collagen scaffold (HC) and their morphology and beating rates compared with ones on conventional plastic dishes. On the first day, the cells attached to HC had already started beating. As time went on, the rate of beating increased as the pores of HC gradually filled with the cells, which integrated to form the cell–matrix complex. At day 8, beating reached the highest frequency of 162 beats per minute, which was twice that of the control cells on plastic dishes. It was concluded that 3-D geometry of the HC is conducive to functional growth of the myocardial tissues, and will potentially be useful for tissue engineering of myocardial regeneration. **Key Words:** Honeycomb collagen—Myocardial cells—Three-dimensional culture—Biocompatibility—Beating rate.

The purpose of this article is to report the establishment of a three-dimensional (3-D) matrix that could potentially enhance the benefit of delivery of cells to the myocardial regions. In order to establish effective methods of tissue engineering, including myocardium regeneration, we must consider five aspects, (i) cells, (ii) matrices, (iii) supply of nutrients, (iv) regulators such as cytokines, and (v) mechanodynamic factors, according to recent insights into tissue engineering (1–3). Among these five, matrices of either natural or artificial materials are relatively more amenable than the other four for clinical use. The 3-D geometric properties of artificial extracellular matrix (ECM) have most influence on cell development, although the physical, chemical, and biochemical properties of the artificial ECM are also important (1–3).

In this study, we chose honeycomb collagen (HC), as a 3-D geometrical matrix for myocardial cell

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culture, because this matrix has been shown to be an effective substrate for osteogenic cells to form bone tissues (4–6). Recently, it was found that HC had a remarkable effect on stem cells in culture, in that it guided the cells to normal development by preventing teratoma transformation (7). The unique geometry of this scaffold has attracted wide attention since it was accidentally developed in 2001. The scaffold is composed of pepsin-treated type I collagen (atelocollagen) which is nonimmunogenic and biodegradable. It is equipped with numerous straight tunnels with semi-hexagonal walls, the diameter of which is roughly controllable from 0.1 to 0.5 mm in the manufacturing process. Because of our previous experience, we hypothesized that HC, with its distinctive geometry, would serve as a highly effective scaffold for myocardial cells.

## MATERIALS AND METHODS

### Isolation and culture of myocardial cells on HC

Ten newborn Sprague Dawley rats of either gender were used. After anesthesia, their hearts were removed. The myocardial cells of the ventricular tissues were separated by enzymatic digestion and mechanical stimulus as previously described (8). The purified cells ($6 \times 10^5$/mL) were inoculated into 24-well culture plates containing discs of HC (2–3 mm thick, 7 mm diameter). Culture was performed using a standard α-minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA), which contained 15% fetal bovine serum (Sankou Junyaku, Tokyo, Japan) and 1% of antibiotics mixture (penicillin-streptomycin, Sigma) at 37°C in a humidified 5% $CO_2$/95% air environment. Observations of cell morphology and beating rhythm of the cultured cells were performed every other day and continued for 20 days, taking samples for transmission electron microscopy (TEM) at days 8, 16, and 20. As a control group, purified myocardial cells were inoculated into culture dishes without HC under otherwise identical culture conditions. At the end of the culture period, the cell–scaffolds composites were fixed, embedded in paraffin, and sectioned for routine histological observation. Sections (10 μm) were stained with hematoxylin and eosin (HE), brilliant green, and Masson's stain.

### Electron microscopy

Samples of each of the cell–HC composites (1 $mm^3$) were subjected to routine fixation and dehydration, embedded in epoxy resin, and ultrathin sections were cut, stained with uranyl acetate, and examined with a Hitachi H-7500 transmission electron microscope. For scanning electron microscope (SEM) samples, samples were fixed, dehydrated, critical point dried (from liquid $CO_2$), and gold sputter coated.

## RESULTS

### Light and electron microscopy of myocardium cell growth on HC

Under the light microscope, the HC prior to culture can be seen to consist of numerous tunnels divided by semi-hexagonal walls, with sizes ranging 200~400 μm in diameter (Fig. 1A,B), as previously described in more detail (4–6). Four hours after inoculating with myocardial cells, phase contrast microscopy showed that myocardial cells began to attach to the collagen walls (Fig. 1C,D). At this stage, the myocardial cells were round, and later they became fusiform.

On day 4, light microscopy of HE-stained sections indicated that a few myocardial cells had begun to grow into the tunnels of the HC. However, TEM showed that some tunnels were filled with a large quantity of cells, among which most were fibroblast-like, while the myocardial cells were comparatively few. Both kinds of cells seemed to be morphologically normal.

On day 8, the number of myocardial cells increased significantly. TEM observations revealed clear mitochondria with no apparent cellular swelling or vacuolization. The edges of the cell membrane attached closely to the scaffold (Fig. 1E) in many places and some cells extended filopodia onto the scaffold; a few cells grew inside the walls of the scaffold.

On day 16, cells attached closely to the walls of the HC, covering them (M in Fig. 1F,H). Under the SEM, the myocardial cells seemed to penetrate into the HC and stretched out many prominences. At the same time, the cells connected with each other, forming a web-like or lamellar structure and also integrated with the scaffold, leaving small spaces between them (Fig. 1F,H). The cells grew from the walls of HC to the center of the tunnels which were eventually completely filled with cells.

On day 20, the cells swelled slightly, and there were vacuoles and evidence of apoptosis. In some samples, the cells attached to both sides of the HC. Adjacent to some fibroblast-like cells, collagen fibrils with striations could be seen. A cross-section of a blood capillary was occasionally seen (Fig. 1E).

**Beating rhythm of myocardial cells on HC**

On the first day, some of the cells on HC were observed to beat occasionally. The beating was accompanied by the scaffold, that is, the entire cell–HC composites were beating. The beating frequency was 16 beats per minute (bpm), and the rhythm was regular gradually increasing to 23 bpm on average, on day 3. At 8 days, the frequency had increased up to 177 bpm. After the 10th day, it slowed, down to 48 bpm at 20 days, as shown in Fig. 2.

In contrast, few beating cells could be found in control dishes on the first day. Their beating frequency was 17 bpm on average, with a regular rhythm. On the eighth day, the beating frequency had reached 90 bpm; it decreased thereafter and was 30 bpm on the 20th day (Fig. 2).

## DISCUSSION AND CONCLUSIONS

It was expected that the collagenous biomaterial would provide a feasible artificial ECM for the growth and differentiation of myocardial cells (9–12), particularly when it has a unique geometry of HC. In this study, higher beating rates were achieved in HC

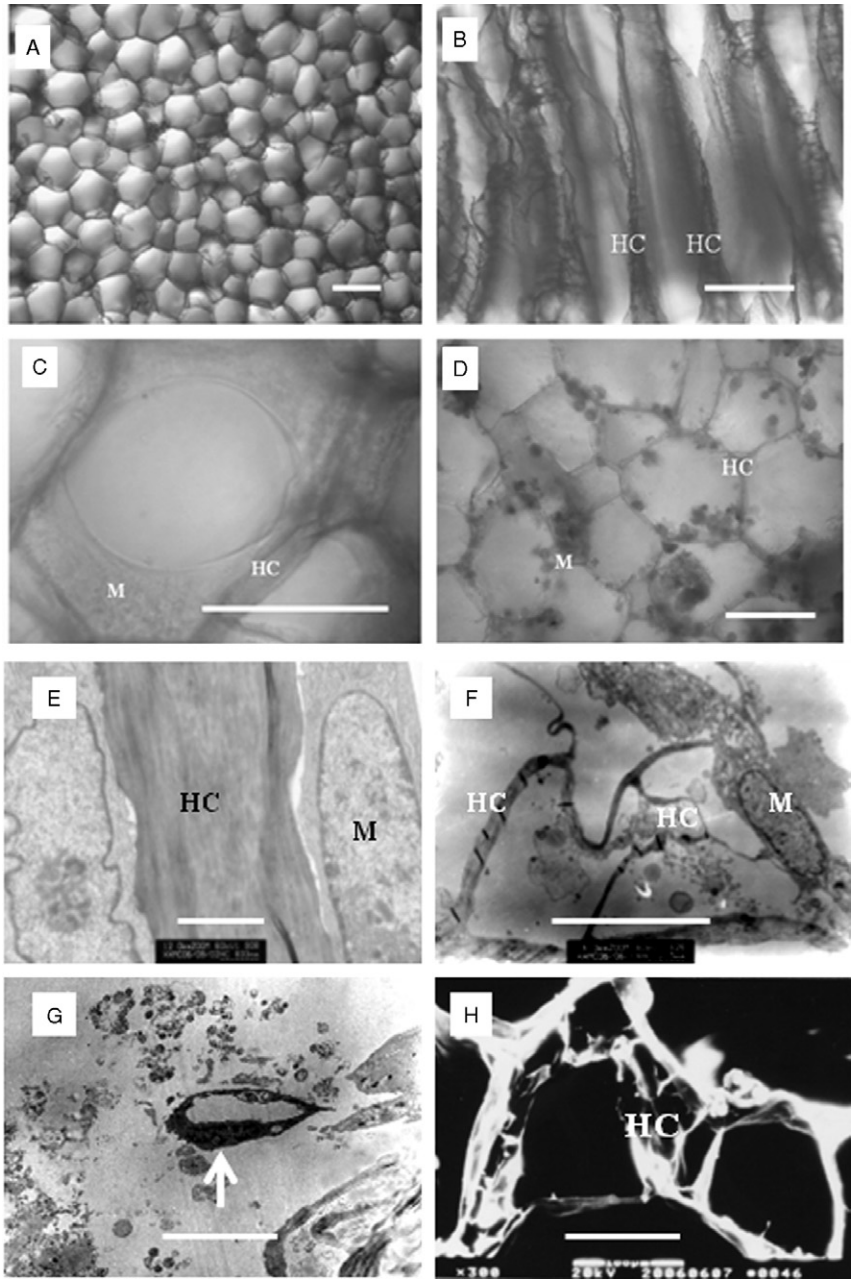


**FIG. 1.** Morphological observations of developmental process of myocardial cells in HC. (A, B) Light microscopy of HC before inoculating cells in a cross section (A) and a longitudinal section (B). (C, D) The myocardial cells were growing closely attaching to the walls of HC at day 4 of culture. HC and M in Fig. 1C,D indicate the wall of HC and myocardial cells, respectively. Bars in all figures indicate 500 μm. (E) Myocardial cells attached to the two sides of the wall of HC. HC: collagen scaffold, M: myocardial cells (TEM × 12 000) at day 8. Bar indicates 10 μm. (F) Myocardial cells of culture, when the cells integrated with the scaffold and filled the HC tunnels (TEM × 4000) at day 16. Bar indicates 50 μm. (G) Blood capillary appeared in the transverse section as indicated by arrow (TEM × 6000) at day 20. Bar indicates 25 μm. (H) The myocardial cells, attaching to the surface of the collagen fibers of HC, integrated with one another (SEM × 1500) on day 16. Bar indicates 10 μm.

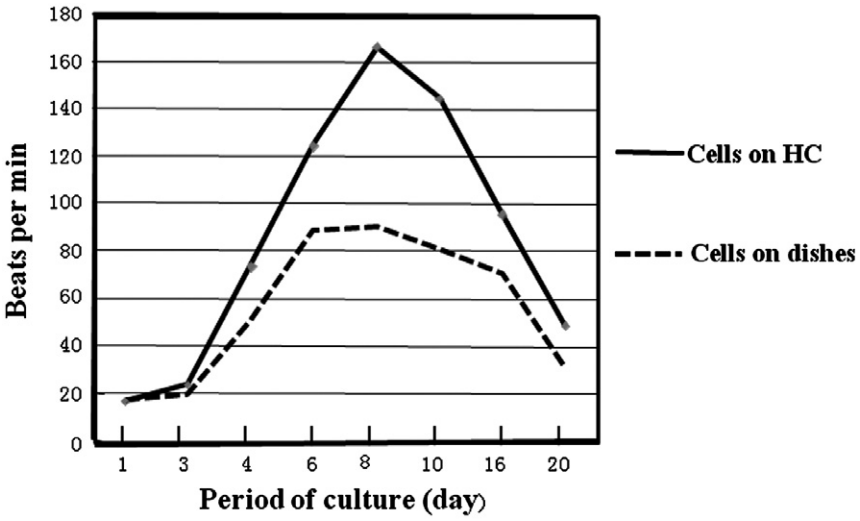


**FIG. 2.** Comparison of beating frequency by myocardial cells cultured on conventional dishes (solid line) and those on HC (broken line).

than in normal cultures probably due to the microenvironments provided by the HC tunnels, whose diameters were close to the optimal pore size reported (1). Some cells also grew into the wall of the scaffold, forming a complex of the cell–collagen scaffold. Under TEM and SEM, it was found that the cells were attaching to the two sides of the walls of HC, and stretched out filopodia to integrate with the scaffold, further confirming the close relationship between the HC and the myocardium. The results indicated that the HC and the myocardial cells were biologically compatible and that HC is a feasible material for 3-D cultures intended for regeneration of myocardial tissues. The advantages of HC over other collagenous (9–12) and noncollagenous scaffolds (13–15) may be summarized as: (i) conductive geometrical structure; (ii) biocompatibility; and (iii) biodegradability as in natural collagen.

Myocardial tissue engineering is a novel and demanding science (16). In the next steps in the development of the technology, we must consider not only the geometry of scaffolds such as that of honeycomb but also mechanodynamic devices (11) or bioreactors (17), which provide the cells with an environment similar to that of the human body, particularly the circulation of medium into the inner portion of the 3-D cell–matrix composites. These devices are also being made in our laboratory.

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